THE SIZE, SHAPE AND HYDRATION OF LOBSTER HEMOCYANIN [1]

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Sedimentation (Eriksson-Quensel and Svedberg, 1936), diffusion (Polson, 1939a), partial specific volume (Svedberg, 1939), intrinsic viscosity (Polson, 1939b), and sedimentation equilibrium (Eriksson-Quensel and Svedberg, 1936) studies have been carried out on the hemocyanin of the lobster, *Homarus vulgaris*. While these studies serve in a general way to characterize the hemocyanin molecule, several important pieces of information are lacking. First, sedimentation coefficients were not extrapolated to zero concentration. Second, no information concerning hydration was provided. Third, no independent evidence of shape was presented. The purpose of the present study is to provide a more precise and more complete characterization of the lobster hemocyanin molecule.

A. Preparation of Hemocyanin

North American lobsters, *Homarus americanus,* obtained in Woods Hole, Massachusetts, were used in this study. The technical descriptions of the species appearing in Milne-Edwards (Milne-Edwards, 1837) permit the conclusion that *H. americanus* and *H. vulgaris* are very similar if not actually indistinguishable. The lobsters were bled, the blood was defibrinated by whipping, and the hemocyanin was purified by alternate centrifugation for ten minutes in a Servall angle centrifuge at 5000 rpm and for six hours in a type 40 rotor in a Spinco Model L centrifuge at 35,000 rpm. All centrifugations were carried out in the cold. Two low speed and one high speed runs were used to prepare the hemocyanin for diffusion and sedimentation experiments. Several additional cycles of low and high speed centrifugation were used in the preparation of material for partial specific volume, intrinsic viscosity, and specific refractive increment measurements.

B. Sedimentation Studies

1. *Sedimentation as a function of concentration*

Sedimentation studies on various concentrations of hemocyanin in $M/10$ phosphate buffer at pH 7 were carried out in a Spinco ultracentrifuge. Solution densities were measured with a pycnometer. Hemocyanin concentrations were determined by means of a refractometer, using 0.00193 as the specific refractive increment. Solution viscosities were measured at a temperature below and at a temperature above that of the sedimentation run, and the value at the tempera-

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TABLE I

Sedimentation of lobster hemocyanin at various concentrations

Hemocyanin concentration (g/100 ml.)	s Svedbergs	η Solution viscosity (cps)	ηs	ηs corrected	η_0 Solvent viscosity (cps)	$\eta_0 s$	$\eta_0 s$ corrected	Solution density
10.7	7.65	2.1454	16.4	20.1	1.0439	8.0	9.8	1.053
4.77	14.1	1.4008	19.8	23.0	1.0305	14.6	16.9	1.039
2.75	18.0	1.2179	21.9	24.5	1.0362	18.7	20.9	1.029
1.55	20.1	1.0982	22.0	24.1	1.0057	20.2	22.1	1.024
1.5	20.9	1.0156	21.2	23.2	0.9000	18.8	20.6	1.024
0.5	22.8	0.9859	22.5	24.3	0.9200	21.0	22.7	1.020
0.0				24.5			24.5	

ture of the sedimentation run was determined by interpolation. Solvent viscosities were determined in the same way. The product of sedimentation coefficient and solution viscosity and the product of sedimentation coefficient and solvent viscosity were then each corrected for solution density by multiplying by

$$(1 - V_h' \, d_w^{20}) \; / \; (1 - V_h' \, d),$$

where V_h' has the meaning defined in the next section, d_w^{20} is the density of water at 20°, and d is the density of the solution investigated in the ultracentrifuge. The figures thus obtained are identified by the symbols $\eta \; s$ (corr) and $\eta_0 s$ (corr), respectively. The data are listed in Table I.

It was found that viscosity-sedimentation products gave more nearly linear plots with concentration than did reciprocals of viscosity-sedimentation products. In the case of most virus characteristic particles, solution viscosity-sedimentation products are essentially invariant with concentration (Lauffer, 1944; Lauffer and Stanley, 1944; Taylor, Epstein and Lauffer, 1955). This was not found to be the case with lobster hemocyanin. Solution viscosity-sedimentation products, and solvent viscosity-sedimentation products were extrapolated to the same value at zero concentration. This value is listed in Table I. When one divides it by the viscosity of water at 20°, 0.01005, one obtains the sedimentation coefficient, corrected to water at 20°, at infinite dilution. This has a value of 24.5×10^{-13} cgs units. The previously reported value, 22.6×10^{-13} (Eriksson-Quensel and Sved-

TABLE II

Sedimentation of lobster hemocyanin in sucrose solutions

s Svedbergs	η Solution viscosity (cps)	ηs	d Solution density
18.85	1.158	21.8	1.0314
11.9	1.555	18.5	1.0587
7.0	2.196	15.4	1.1027
3.28	3.727	12.2	1.1445
1.05	8.287	8.7	1.1909
0.22	19.793	4.4	1.2420
0.06	25.463	1.4	1.2719

berg, 1936) is the mean of the values obtained from various dilutions of lobster blood in different electrolyte solutions; it does not represent the value at infinite dilution.

2. Sedimentation in sucrose solutions of various densities

Sedimentation studies on 0.9% hemocyanin in 0.1 *M* phosphate buffer at pH 7, to which various amounts of sucrose had been added, were carried out. Sedimentation coefficients, solution viscosities and solution densities were evaluated as described previously. The data are recorded in Table II. The product of solution viscosity and sedimentation coefficient, not corrected in any way, was plotted against solution density. The data fit the straight line, $\eta s \times 10^{15} = 104.7 - 80.9d$. The intercept at a density of 0.998, the density of water at 20°, is 24.0×10^{-15}. This is in good agreement with the solution viscosity-sedimentation coefficient product for 0.9% hemocyanin solution, 24.2×10^{-15}, as interpolated from the data of Table I. The intercept corresponding to zero sedimentation rate occurs at a

TABLE III

Viscosity at various concentrations

Concentration (g/ml.) C	Concentration (volume fraction) $\phi = CV$	Relative viscosity η_r	$\frac{\eta_r - 1}{\phi}$
0.107	0.0792	1.9881	12.5
0.0477	0.0353	1.3259	9.25
0.0275	0.0204	1.1448	7.1
0.0155	0.0116	1.0713	6.15
0.0083	0.0062	1.0300	4.85

density value of 1.294. The reciprocal of this value, 0.773, is taken to be the effective specific volume of the hydrated hemocyanin molecule, V'_h (Lauffer, Taylor and Wunder, 1952).

C. DIFFUSION EXPERIMENTS

The diffusion coefficient of an 0.9% hemocyanin solution in 0.1 *M* phosphate buffer at pH 7 was determined at 20° in a Perkin-Elmer electrophoresis apparatus. A value not significantly different from that reported by Polson (Polson, 1939a) was obtained. Because Polson's data are more extensive, his value for D_w^{20} of 2.77×10^{-7} cm.^{2}/sec. will be used in subsequent calculations.

D. INTRINSIC VISCOSITY

Viscosities of solutions of various concentrations of hemocyanin in 0.1 *M* phosphate buffer at pH 7 were determined using a capillary viscometer in a water bath at 25°. Concentrations were measured by means of a refractometer using a value for specific refractive increment of 0.00193. The data are shown in Table III. Intrinsic viscosities or viscosity numbers were evaluated by plotting the quotient of relative viscosity increment divided by volume fraction against

volume fraction and extrapolating to zero. A value of 4.85 ml. per ml. was obtained. Polson (Polson, 1939b) reported a value of 6.4. The value obtained in the present study, namely 4.85, is used in subsequent calculations.

E. Partial Specific Volume

The apparent partial specific volume of lobster hemocyanin was calculated from the dry weight composition and the density of an aqueous solution of a highly purified preparation. A mean value not significantly different from that for the partial specific volume reported by Svedberg (Svedberg, 1939), namely 0.74, was obtained.

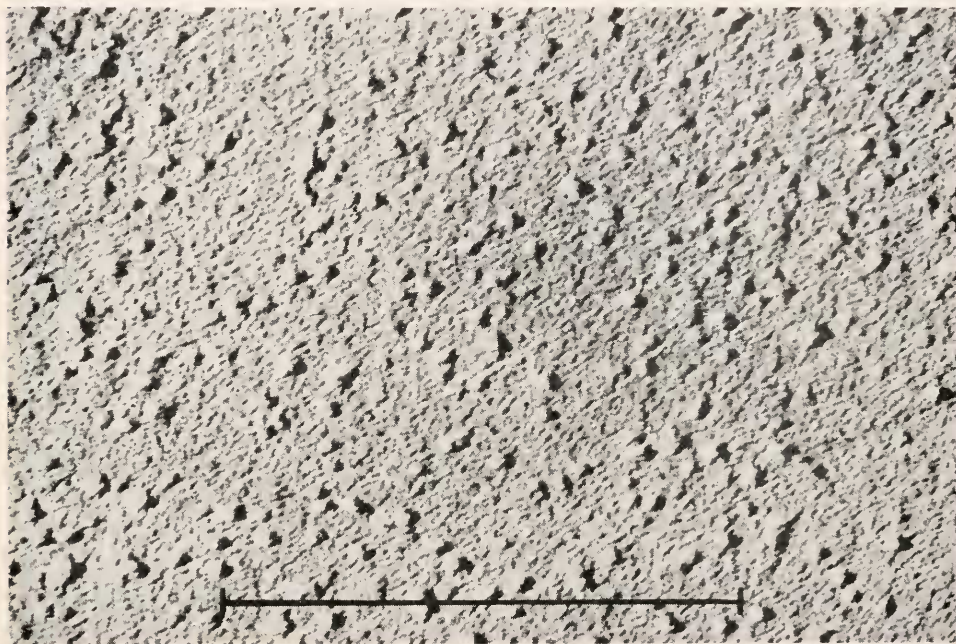

Figure 1. Electron micrograph of hemocyanin molecules. Uranium shadowed (× 69,000). Shadow angle slope — ¼. Line corresponds to one micron.

F. Specific Refractive Increment

The specific refractive increment was determined on aqueous solutions of highly purified hemocyanin by means of a Phoenix Precision differential refractometer. The concentration was determined by dry weight. The specific refractive increment obtained was 0.00193. Redfield had previously reported a value of 0.002 (Redfield, 1934).

G. Electron Microscopy

Electron micrographs of hemocyanin molecules shadowed with uranium were kindly made by Dr. Irwin Bendet of the Department of Biophysics at the Uni-

versity of Pittsburgh. A representative micrograph is shown in Figure 1. The electron micrograph shows clearly that the hemocyanin molecules are not rod-shaped. The shadow lengths are consistent with a height of 7.5 mμ and the modal image size corresponds to a particle with a diameter of 33 mμ. Thus, the micrograph is consistent with the assumption that the hemocyanin molecule is a flattened ellipsoid of revolution about 33 mμ in diameter and 7.5 mμ thick. Naturally, these dimensions refer to dried particles.

Discussion

The following parameters, determined in the present and in previous investigations, can be used to calculate the size, shape, and degree of hydration of lobster hemocyanin; sedimentation coefficient, $s_w^{20} = 24.5 \times 10^{-13}$; diffusion coefficient, $D_w^{20} = 2.77 \times 10^{-7}$; partial specific volume, $V = 0.74$; effective specific volume of the hydrated particle, $V_h' = 0.773$; intrinsic viscosity or viscosity number, $[\eta] = 4.85$. Details of these calculations have been reviewed recently (Lauffer and Bendet, 1954).

From the sedimentation coefficient, the diffusion coefficient, and the partial specific volume, the molecular weight and the friction ratio can be calculated to be 825,000 and 1.25, respectively. This molecular weight value supersedes the value of 752,000 reported by Eriksson-Quensel and Svedberg (Eriksson-Quensel and Svedberg, 1936) and compares with their sedimentation equilibrium value of 803,000. From the partial specific volume, V, and the effective specific volume of the hydrated particle, V_h', one can calculate that hemocyanin is hydrated to the extent of 0.15 ml. of water per g of anhydrous protein. The reservations which must attend the use of hydration values calculated in this manner have been discussed previously (Lauffer, Taylor and Wunder, 1952; Lauffer and Bendet, 1954).

If it is assumed that the molecule is a flattened ellipsoid of revolution and that its hydration is actually equal to the value calculated from sedimentation vs. medium density studies, namely, 0.15 ml. of water per g protein, then one can interpret the friction ratio, 1.25, to be consistent with a hydrated flattened ellipsoid of revolution with an axial ratio of 4.14, corresponding to a diameter of 32.2×10^{-7} cm. and a thickness of 7.8×10^{-7} cm. These values are consistent with those estimated from the electron micrograph. The axial ratio of the hydrated particle calculated on the basis of the same assumptions from the intrinsic viscosity was 4.05, in good agreement with the value obtained from sedimentation, diffusion and partial specific volume data.

Summary

1. The sedimentation coefficient of lobster hemocyanin corrected to water at 20° and extrapolated to zero concentration has been found to be 24.5×10^{-13} cgs units.

2. The intrinsic viscosity has been found to be 4.85 ml./ml.

3. From the variation of the sedimentation rate of hemocyanin in sucrose solutions of different densities and from the partial specific volume, the hydration of hemocyanin was calculated to be 0.15 ml. of water per gram of dry protein.

4. The specific refractive increment was determined to be 0.00193 ml. per 10^{-2} g.

5. From the above parameters and others taken from the literature, lobster hemocyanin molecules were calculated to have a molecular weight of 825,000, corresponding to oblate ellipsoids of revolution with hydrated diameter of 32.2 mμ and hydrated thickness of 7.8 mμ.

ERRATUM

AUTHORS' ERROR. Lauffer and Swaby, Vol. 108, pp. 294 and 295.

The dimensions of the lobster hemocyanin molecule, considered as a flattened ellipsoid of revolution hydrated to the extent of .15 ml. water per g. protein, were erroneously reported as 32.2×10^{-7} cm. for the diameter and 7.8×10^{-7} cm. for the thickness. The correct values are 21.4×10^{-7} and 5.2×10^{-7} cm. These are not very close to the electron microscope values. Therefore, the question of the interpretation of the hydrodynamic results is re-opened.

An additional possibility is that the hemocyanin molecules are spheres. If so, the friction ratio of 1.25 must be ascribed to hydration alone. By methods described by Lauffer and Bendet (1954), one can calculate a value of .70 ml. of water per g. of protein from this friction ratio. A sphere composed of protein with partial specific volume of .74, with an anhydrous molecular weight of 825,000 and with this degree of hydration, would have a diameter of 15.6×10^{-7} cm. Such a particle could collapse into a disk upon drying, but its total volume would be less than one-fourth that indicated by the electron micrographs. The new value for hydration is considerably higher than that calculated from the sedimentation vs. density experiments. As was pointed out in a reference cited in the discussion, however, the value calculated from sedimentation vs. density experiments represents only the water in excess of that associated with other constituents in the hypothetical hydrating medium and might underestimate the total.

If one takes the dimensions indicated by the electron micrograph at face value, the particles seen in the micrograph cannot be interpreted as single hemocyanin molecules, for their volumes are much too great. However, if the thickness of the uranium coat is taken into account, the resulting dimensions yield a volume which may not be inconsistent with the hydrodynamic data.